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13. ABSTRACT *(Maximum 200* The purpose of this grant was to support predoctoral training on an experimental model relevant to breast cancer etiology. Specifically, we explored the multifactorial nature of the interaction of a growth factor (TGFalpha, which is commonly expressed,both in benign and malignant proliferative disease of the human breast)and an oncogene (c-myc,whose gene is amplified and whose protein is inappropriately expressed in 20-30% of human breast tumors. Using a transgenic mouse model, we observed that co-expression of these two genes was remarkably synergistic for onset and progression of mammary tumors. Using in vitro culture of explanted, single and bitransgenic mouse mammary tumor cells, this project explored TGFalpha-myc synergy at the dual levels of cell survival and proliferation. While myc induced both p53 and bax death-promoting genes, TGFalpha promoted cell survival by inducing BclXL. In terms of the cell cycle, myc shortened G1 by modulating cyclin E, p27, and cdc25A, which activated cdk-2 and inactivated Rb. Thus, the interaction of TGFalpha and myc promoted shortened, aberrant cycles resulting in survival of genetically aberrant cells.

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INTRODUCTION

The c-myc proto-oncogene is frequently amplified in many human cancers including breast cancer, and its amplification is associated with a high proliferation rate and poor prognosis (1-4). Transgenic animal models have confirmed the tumorigenic potential of c-myc overexpression in mammary tissue (5-9), but the mechanism by which Myc promotes tumor growth has not been elucidated. De-regulated c-myc expression can promote cell transformation in cooperation with growth factors such as EGF (10), and has been associated with cell proliferation as well as apoptosis (11, 12). Thus, the experiments described here were intended to identify components of the pathways through which Myc acts to both increase proliferation and induce apoptosis in mammary epithelial cells and to examine the impact of EGF on those pathways. To explore these mechanisms, this project initially set out to characterize effects of Myc on cyclins regulating the G_1-S phases of the cell cycle. However, in the first year of our study it became evident that the effects of Myc were not mediated primarily through cyclin regulation. Our studies have focused instead on characterization of other regulators of the cell cycle (cdk inhibitors and cdk phosphorylation state) and cell death (bcl family of proteins). The studies have primarily utilized two mammary epithelial cell lines (MECs): 1). Myc #83 cells (13) which were derived from a mammary tumor of a Myc transgenic mouse and can be induced to undergo apoptosis by altering their growth environment (i.e. removal of the growth/survival factor EGF or addition of the growth inhibitor TGFβ). 2). A1N4-myc cells (14) which are chemically immortalized human MECs which demonstrate an accelerated growth rate compared

to parental cells but which undergo reversible growth arrest rather than apoptosis in the absence of EGF.

By using these two types of mammary cell lines, both aspects of Myc overexpression have been examined. In Year 2, we completed our studies of apoptosis concluding that Myc upregulates the death protein Bax; this effect is countered by EGF (TGFα) induced $BclX_L$. We also found that Myc serves to modulate Rb phosphorylation through activation of cdk-2, secondary to a large decrease in p27 and a smaller induction of cyclin E. In the third year, we have continued these cell cycle studies to address possible modulation of cdk-2 phosphorylation status and to begin to set up a system in which to regulate Myc.

BODY

Materials and Methods

Cell lines

The benzo(a)pyrene-immortalized and transformed human mammary epithelial cell line 184A1N4-myc (10,14) and its parental cell line 184A1N4 were used to study the effects of myc overexpression on cell cycle regulation. The A1N4-myc line was established *via* retroviral infection with a construct containing mouse myc under the control of the Moloney mouse leukemia virus long terminal repeat (MMLV LTR). Both cell lines were maintained in media containing 0.5% fetal calf serum (FCS), 0.5μg/ml hydrocortisone, 5μg/ml insulin, and 10 ng/ml epidermal growth factor (EGF, Upstate Biotechnology Incorporated, Lake Placid, NY). The cells arrest in G_1 in the absence of EGF.

RNA Isolation

A1N4 and A1N4-myc cells were plated sparsely (1.5×10^6 cells) in culture flasks (225 cm^2; Costar) and growth arrested as described above. Following re-stimulation with EGF (10ng/ml), total RNA was harvested at three hour intervals by the guanidine thiocyanate-acid phenol method (15).

Northern analysis

Total RNA (12μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were hybridized overnight with a 700bp ^{32}P-labeled, random-primed human probe for cdc25A (nt936-1637). Bands were detected with a PhosphorImager 445 SL.

Myc-ER Inducable Construct

We obtained a *c-myc-ER* expression construct and an inactive vector control *c-delta myc ER* from M.J. Bishop (UCSF, San Francisco, CA). Initial transfections of A1N4 cells are using Lipofectamine (Life Technologies Inc, Gaithersburg, MD), according to the manufacturers recommendations. Backup strategies will include electroporation, calcium phosphate-mediated transfection, and the use of an alternate, non-transformed mammary epithelial cell line, MCF10A (initially provided by the Michigan Cancer Foundation, now also available through ATCC, Rockville, MD).

Results

Myc and cell cycle regulation

We previously showed that c-myc overexpression in both mouse (HC-14-myc and MMEC-myc) and human (A1N4-myc) MECs decreased their doubling time by about 6 h, compared to parental lines. Experiments with the A1N4 lines suggested that this difference was not due to increased sensitivity to EGF, but rather to a shortening of G_1. A1N4 and A1N4-myc cells were arrested in G_1 in the absence of EGF and were allowed to re-enter the cell cycle by replacing EGF. FACS analysis demonstrated that the A1N4-myc cells began to enter S phase 12h after EGF addition and percent cells in S phase peaked at 18h. In contrast, parental cells did not enter S phase until 18h and peaked at 24h.

The shortened G1 phase does not appear to be due to any gross changes in cyclin A or D1 RNA expression as assessed by a non-radioactive RNase protection assay, which we have developed (17). In unsynchronized cells, Myc overexpression had no significant effect on cyclin A or D1 mRNA expression. Cyclin mRNA was nearly undetectable in arrested cells and

induction was closely correlated with changes in cell cycle phase. However, subsequent studies provided strong support for multifactorial regulation of cdk-2 by Myc. First, we demonstrated that the Rb protein appeared to be permanently phosphorylated throughout the cycle and unaffected by withdrawal of EGF (coupled to cycle arrest). Second, we found that this effect was paralleled by a cell cycle-independent activation of cdk-2. Cdk-2 activation appeared to be secondary, in part, to a modest induction of cyclin E and to a strong suppression of expression of p27. The activational CAK phosphorylation also appeared to correspond to the catalytic activation of cdk-2.

New Results - cdc25A regulation

In the third year, we have begun to examine the determinants of inhibitory phosphorylation of cdk-2. Specifically, we studied regulation of the oncogenic phosphatase cdc25A, since it is thought to be a major determinant of cdk-2 activity and since a recent study identified the cdk2 phosphatase cdc25A as a direct transcriptional target of Myc. In unsynchronized cells, cdc25A RNA was elevated compared to parental cells (Figure 1A). The result was somewhat unexpected since a similar analysis of cyclins A and D1 RNA showed no significant differences between the two cell lines during asynchronous growth (not shown). However, despite the elevated RNA levels, the time of cdc25A induction in synchronized cells was similar in both cell lines, beginning at 12h and reaching maximum levels between 15 and 21h after EGF stimulation (Figure 1B).

New results - Cell transplantation with regulatable Myc-ER

In the third year, we have also worked toward developing a system whereby we may regulate expression of Myc in our model system. The purpose of such studies is to assess the

temporal relationships among Myc, its two putative regulators p27 and cyclin E, activation of cdk-2, and phosphorylation (inactivation) of Rb. Of two available approches; transcriptional regulation under Tet operon (18), and post transcriptional regulation of an estrogen receptor-Myc chimera (17), we have chosen the latter. The reasons for this choice were its immediate availability and the extremely rapid time-course experiments it could afford.

We are currently attempting electroporation, calcium phosphate, and lipofectamine procedures to transfect the A1N4 cells with the Myc-ER construct we have obtained. Should transfection of these cells prove to be problematic, we will use the human MCF-10A mammary epithelial cells (obtained from Michigan Cancer Foundation) as a back-up. These cells have similar, EGF-dependent regulation of the cell cycle compared to A1N4 and they have been easily transfected by other laboratories (data not shown). We hope that these experiments will be completed by September of this year.

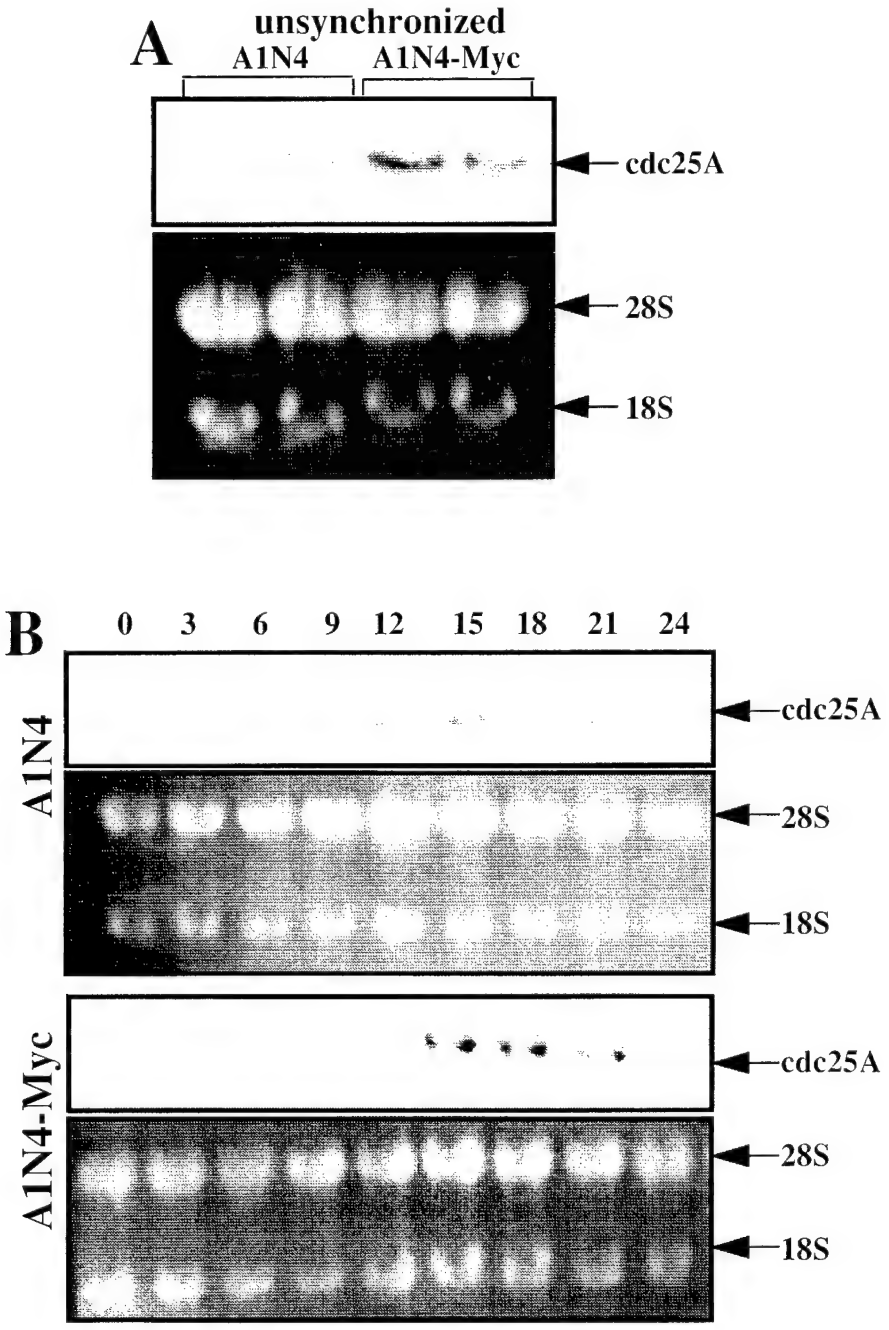

Figure 1: Northern analysis pf cdc25A RNA in A1N4 and A1N4-myc cells. **A:** Expression in unsynchronized cells. Cells were grown in the presence of EGF and were harvested at approximately 75% confluence. **B:** Cell cycle dependent expression. Cells were arrested and re-stimulated by addition of EGF. At the times indicated, total RNA was harvested.

CONCLUSIONS

Regulation of the cell cycle by Myc

The cdc25 family of phosphatases has been implicated in the regulation of cdk activity, since its members remove inhibitory phosphate groups from serine 14 and tyrosine 15 of cdks (19). In particular, evidence of this effect has been clearly demonstrated for cdk-2. This is less clear for cdk-4; although UV irradiation stimulates tyrosine phosphorylation of cdk4 with subsequent G_1 arrest (20), a clear function for such a cdk4 species in normal cell cycle progression has not been demonstrated. Thus, cdc25 expression may not be as important for cyclin D1/cdk4 activity as it is for cdk2 activation.

The A and B forms of cdc25 can function as transforming oncogenes in cooperation with activated Ha-ras or with the loss of Rb (21). The synergism between Ras and cdc25 may be explained by the observation that Raf1, a component of the Ras signal transduction pathway, can phosphorylate and activate cdc25 proteins (22). The assertion could also explain the results of Steiner et al., (23). They found that full induction of cdk2 kinase activity by Myc also required cdc25A activity, which could apparently be stimulated by serum growth factors, perhaps through the Ras-Raf pathway. A more recent study indicates that cdc25A expression can be directly induced by Myc in fibroblasts (24). However, another study using a similar rat fibroblast cell line showed no increase in cdc25A steady state levels when Myc was overexpressed (25). In our MEC system, cdc25A RNA levels were elevated by Myc overexpression, but the timing of cdc25A expression induction following exposure to EGF was quite similar in the two MEC lines. Its RNA was first detected 12 hours after EGF addition, suggesting that other factors in addition to Myc are required for cdc25A expression.

Figure 2 summarizes the results of this grant so far. The major effects of Myc appear to be inhibition of expression of p27 and activation of cyclin E. However, cdc25A appears to be under controls in addition to Myc. Through potentially multiple influences, Myc thus activates cdk-2, which inactivates Rb. Thus, cells are poised in G_1 to respond to EGF with a shortened, aberrant cell cycle.

Current and Future Studies

Our current studies focus on evaluation of the temporal relationships among the proposed steps in Myc regulation, as depicted in Figure 2. We hope to refine our hypothesis by examining its close relationship among Myc induction, one (or more) of its mediators (p27, cyclin E, cdc25A), and subsequent, downstream effectors (such as Rb).

Our longer range studies (now beyond the scope of the grant) entail establishing more cause and effect relationships among dysregulation of cyclin E, p27, cell cycle shortening, and tumorigenesis. First, we hope to obtain cyclin E and p27 expression constructs. These will be transfected into A1N4 (or MCF10A) cells to assess their ability to mimick Myc and shorten the G1-phase. Next, we hope to obtain an MMTV promoter-driven cyclin E transgenic mouse strain and a p27 knockout mouse strain. Each will be mated to our TGFα transgenic strain to establish the scope of each alteration for interaction with TGFα in a synergistic fashion (like Myc) for mammary tumorigenesis.

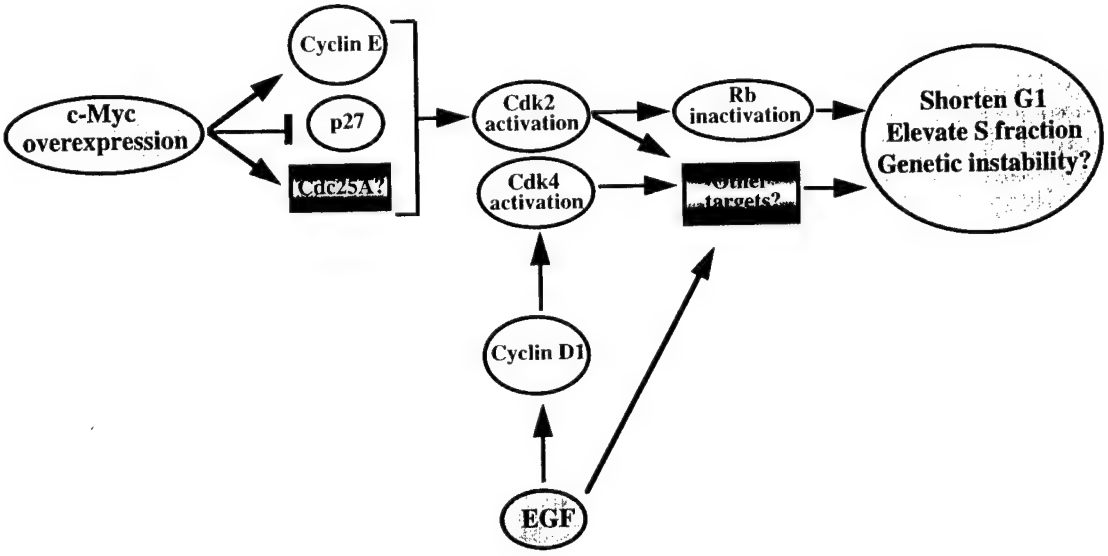

Figure 2. Proposed model for the effects of c-myc overexpression on cell cycle regulation in MECs. Myc induces cdk2 activation, via increased cyclin E expression and decreased p27 expression, which in turn can alter the phosphorylation state of Rb. Myc may also induce Cdc25A expression, which would further stimulate cdk2 activity. EGF is required for cyclin D1 expression. Additional targets of the EGF receptor signaling pathways may also be necessary for progression into the S phase. The combination of c-myc overexpression and EGF signaling leads to accelerated proliferation and may therefore promote genetic instability. (See Ref 26)

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APPENDIX

Manuscript submitted to Cancer Research

ALTERED CELL CYCLE REGULATION IN MAMMARY EPITHELIAL CELLS WHICH OVEREXPRESS c-MYC[1]

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Running Title: Myc and and the cell cycle in breast cancer

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Abstract

The c-*myc* gene is commonly amplified and/or overexpressed in primary human breast cancers, and mouse transgenic models have demonstrated that c-*myc* expression can play an important role in mammary tumorigenesis. We investigated cell cycle control in mammary epithelial cells (MECs) with constitutive c-*myc* expression. In both mouse and human MECs, c-*myc* overexpression decreased the doubling time by about 6 h compared to parental lines. The decrease was not due to a change in EGF sensitivity, but rather to a shortening of the G_1 phase of the cell cycle. Rb was constitiutively hyperphosphorylated in cells with exogenous Myc expression, in contrast to the parental cells which exhibited a typical phosphorylation shift as they traversed G_1. The abnormal phosphorylation status of Rb in c-*myc*-overexpressing cells was associated with premature activation of cdk2 kinase activity as a result of reduced p27 expression and elevated cyclin E expression.

Introduction

The proto-oncogene c-*myc* encodes a highly conserved nuclear phosphoprotein which contains a leucine zipper and a basic-helix-loop-helix motif common to many transcription factors (1-5). When bound to its heterodimeric partner Max, Myc protein binds specifically to DNA and can activate transcription. However, the specific targets of *myc* regulation are not well characterized, and thus its mode of action is poorly understood, despite intense investigation.

Myc has been implicated in the regulation of cell proliferation, differentiation, and death by apoptosis (reviewed in 1-6). Since aberration of any of those normal processes can contribute to tumorigenesis, it is not surprising that deregulated expression of the c-*myc* gene is often associated with neoplasia. *In vitro*, c-*myc* overexpression can cooperate with other oncogenes such as Ras to transform cells. Additionally, the ability of inappropriately expressed Myc to promote tumorigenesis *in vivo* has been clearly demonstrated by transgenic mouse models (7)

Classified as an immediate early gene, c-*myc* expression is tightly regulated and correlated with the proliferative state of the cell (8). In normal quiescent cells, Myc protein levels are very low and its expression is strongly induced following mitogen stimulation. Similarly, expression decreases as cells become growth arrested or undergo differentiation. A reduction in c-*myc* levels due to disruption of one copy of the gene results in a lengthened G_1 cell cycle phase (9), while inhibition of c-*myc* expression blocks cell cycle progression and leads to G_1 arrest (10-11). Conversely, when c-*myc* expression is deregulated, cells are often unable to withdraw from the cell cycle when signaled to do so (12-13).

Based on the above observations, c-*myc* has long been thought to control key aspects of the proliferative response. Since passage through the cell cycle is orchestrated by the cyclins and their associated cyclin dependent kinases (cdks, reviewed in 14), those regulatory proteins would be logical targets for such a proposed action of Myc. Normally, expression of the various cyclins is tightly regulated and is characteristic of specific stages of the cell cycle. Several studies in fibroblasts and hematopoietic cells in fact suggest that expression or activity of some cyclins (ie.

cyclins D1, E, and A) and cdks (ie. cdks 1 and 2) may be altered by c-*myc* expression (9, 15-20). In addition to the activating cyclin subunits, cdk activity can be modulated by cdk inhibitors as well as by a number of kinases and phosphatases (reviewed in 21), some of which have also implicated as targets of Myc (20 [p27]; 22 [Cdc25A]). However, with the exception of the cdc25A gene, the genes in question lack Myc-Max consensus binding sites in the promoter region, indicating that their regulation by Myc is indirect.

Although some mechanistic details of the action of Myc have emerged from studies with rodent fibroblasts, there is considerable interest in further elucidating the mechanisms(s) of malignant transformation by Myc in human epithelial and hematological malignancies. Overexpression of c-*myc* is thought to play a role in the development of breast cancer since it is commonly amplified and/or overexpressed in human breast tumors (reviewed in 23). Amplification of the c-*myc* gene is often associated with highly proliferative tumors and poor prognosis. In addition, Myc confers tumorigenicity when overexpressed in the mammary gland of transgenic mice. Recent results from our laboratory (24) and others (25) showed that overexpression of TGFα can strongly synergize with c-*myc* in transgenic mice to promote mammary tumor development, confirming previous *in vitro* observations that Myc can cooperate with growth factors such as TGFα or EGF to transform mammary epithelial cells (MECs) (26, 27). The contribution of TGFα may be due, at least in part, to the suppression of Myc-induced apoptosis *via* increased expression of Bcl-x_L (28, 29). However, tumors and cell lines derived from the double transgenic mice also showed an accelerated growth rate compared to those from single transgenic mice (24, 28). Since Myc has been implicated in cell cycle regulation of fibroblasts, the tumorigenic action of constitutive c-*myc* expression in the mammary gland may also be due to aberrant cell cycle progression. Although a variety of changes in the expression of cell cycle regulators have been identified in human breast cancer cell lines and primary tumors (reviewed in 30), little is known about the causes or consequences of cell cycle deregulation in breast cancer. Thus, the purpose of this study was to identify changes in cell cycle regulation in MECs which overexpress c-*myc*, and to examine the impact of EGF on those cells.

Results

We began our studies by comparing the effect of constitiutive Myc expression on the growth rate of human and mouse mammary epithelial cell lines. In both mouse (HC14-myc) and human (A1N4-myc) MECs, c-*myc* overexpression decreased the doubling time by about 6 h compared to parental lines (Table 1). Our results are also in agreement with previously published data which indicated a similar decrease in doubling time by MMLV LTR-driven c-*myc* overexpression in MMEC cells, another cell line derived from normal mouse mammary tissue (26, Table 1). In order to determine whether the faster growth rate was simply due to increased sensitivity to growth factors, the two human cell lines were grown in the presence of various concentrations of EGF for three days. The two resulting dose-response curves were parallel, with the A1N4-myc cells growing faster than the parental cells at all concentrations tested (Figure 1).

The A1N4 and A1N4-myc cells were used to further investigate the observed change in growth rate. In the absence of EGF, neither the parental nor the c-*myc*-infected cell line showed significant growth (Figure 2A). That observation was due to the fact that both cell lines arrested in G_1 upon EGF deprivation (Figure 2B). In order to determine the kinetics of cell cycle progression in the two lines, cells were arrested in G_1 in the absence of EGF and were allowed to re-enter the cell cycle by replacing EGF. FACS analysis demonstrated that the A1N4-myc cells began to enter S phase 12 h after EGF addition and percent cells in S phase peaked at 18 h (Figure 3). In contrast, parental cells did not enter S phase until 18 h and peaked at 24 h. The results suggested that the difference in doubling time was due to a shortened G_1 phase of the cell cycle.

Since Rb is believed to play an important role in the G_1 phase of the cell cycle, we next examined Rb expression and phosphorylation and observed a significant difference between the two cell lines. In arrested A1N4 cells, Rb expression was relatively low and the protein was present only in the hypophosphorylated state (Figure 4). About 6 hours after EGF stimulation, approximately 50% of the protein was found in the hyperphosphorylated state. At all time points beyond 6 hours, Rb protein levels were greatly increased and most of the protein was

hyperphosphorylated. In contrast, Rb was highly expressed and phosphorylated at all timepoints tested in A1N4-myc cells.

We then examined the expression of several proteins which are known to be involved in the regulation of G_1 progression and have been implicated in the regulation of Rb function (Figure 5). Cyclin D1 protein expression was very low in arrested cells, was rapidly induced following EGF stimulation, and remained elevated throughout the remainder of the cell cycle. Cyclin D1 levels were maximal at 6 h after stimulation in A1N4 cells, and at 3 h in A1N4-myc cells. Cyclin E protein was detectable in arrested cells of both lines, but it was further stimulated by EGF addition and then down regulated later in the cell cycle. Peak levels of this cyclin were observed between 9 and 15 h in parental cells, and from 3 to 6 h in c-*myc*-expressing cells. Expression of two cyclin dependent kinases which interact with cyclins D1 and E were also examined. Cdk4 expression was constant throughout the cell cycle in both cell lines, but protein levels were higher in A1N4-myc cells. Western analysis of cdk2 demonstrated a dramatic mobility shift in the protein due to CAK phosphorylation. In A1N4 cells, the shift was observed about 12 h after EGF addition, while A1N4-myc cells already contained low levels of phosphorylated protein even when arrested, with a maximal shift at about 6-9 h post stimulation. As expected, those mobility shifts corresponded to the time of cyclin E induction. CAK is believed to be constitutively active, but can only phosphorylate cdks which are complexed with a cyclin (21). Finally, expression of a cdk inhibitor, p27, was analyzed. The function or expression of any G_1 cdk inhibitors could potentially be altered in cells with a shortened G_1 phase, but we chose to examine p27 first, since there was precendent for modifed p27 levels in fibroblasts with deregulated c-*myc* expression (20). Protein levels were quite high in arrested A1N4 cells and decreased as the cells progressed through the cell cycle. In contrast, p27 was barely detectable in arrested A1N4-myc cells, and was rapidly eliminated following EGF addition.

The results presented in Figure 5 suggested that differences in cdk2 activity might be responsible for the shortened G_1 phase in c-*myc*-overexpressing cells. We therefore directly examined activation of cdk2 in the cells with an *in vitro* kinase assay (Figure 6). As predicted,

arrested parental cells contained very little active cdk2, and a major increase in activity was observed 12 hours after EGF stimulation, the time at which cyclin E was maximally expressed, p27 levels were reduced, and cdk2 was phosphorylated by CAK. In contrast, cdk2 was active even in EGF-deprived A1N4-myc cells, with maximal activation at 6 h after EGF stimulation.

Since a recent study identifed the cdk2 phosphatase Cdc25A as a direct transcriptional target of myc, we also wished to examine its expression in our MEC system. In unsynchronized cells, cdc25A RNA was elevated compared to parental cells (Figure 7A). That result was somewhat unexpected since a similar analysis of cyclins A and D1 RNA showed no significant differences between the two cells lines during asynchronous growth (not shown). However, despite the elevated RNA levels, the time of cdc25A induction in synchronized cells was similar in both cell lines, beginning at 12 h and reaching maximal levels between 15 and 21 h after EGF stimulation (Figure 7B).

The shortened G_1 phase did not appear to be a consequence of any gross changes in cyclin A or D1 RNA expression (not shown). Cyclin RNA was undetectable in arrested cells and induction was closely correlated with changes in cell cycle phase. In both cell lines, cyclin D1 expression was detectable by three hours after EGF treatment and levels remained relatively constant throughout the cell cycle, in agreement with the results by Western blot. In the A1N4-myc cells, cyclin A RNA expression began about 9 hours after EGF stimulation, with a peak at 18 hours (31).

Discussion

The results presented here show that constitutive, elevated expression of c-*myc* leads to altered cell cycle regulation in MECs, with accelerated passage through G_1. We propose that the faster growth rate of c-*myc* expressing MECs is due to increased cdk2 activity and constitutive phosphorylation of Rb. The elevated cdk2 activity in arrested and synchronized A1N4-myc cells compared to parental cells was associated with increased cyclin E expression and diminished expression of the cdk inhibitor p27.

Control of Cdk2 activity by p27 and cyclin E

In normal cells, p27 protein levels undergo cell cycle dependent oscillations, with highest levels in G_1 (32). The protein is also induced, through translational and posttranslational mechanisms, by several conditions which facilitate G_1 arrest, including high density, or exposure to TGFß or lovastatin (32, 33, 34). Interestingly, Myc overexpression can block TGFß-dependent growth arrest in keratinocytes (35). It is thought that p27 associates with cyclin E/cdk2 until cyclin D levels are high enough to sequester the inhibitor in cyclin D/cdk complexes. p27 may thereby detemine the order of cdk activation by inhibiting cdk2 activity until the cyclin D level (and therefore cdk4 activity) is maximal (30, 34). Our observations therefore indicate that this level of regulation is reduced or eliminated in MECs which overexpress c-*myc*. In the parental A1N4 line, p27 was expressed in arrested cells and was down-regulated following EGF addition, in agreement with a recent report which demonstrated that growth arrest by anti-EGFR antibody involves p27 expression (36). The A1N4-myc cells, in contrast, had markedly reduced p27 protein levels, even in the absence of EGF stimulation.

Our findings are therefore, in part, similar to those observed in density arrested fibroblasts following induction of a regulatable c-*myc* expression construct (20). In that study, Myc activation led to a rapid increase in G_1 cdk activity and subsequent Rb phosphorylation. Since those density arrested cells already contained relatively high levels of cyclins D1 and E, an increase in cyclin expression was neither necessary nor observed prior to Myc-induced cdk activation. Rather, the authors suggested that the change in cdk kinase activity was specifically due to a Myc-dependent decrease in p27 levels. However, our results and those of Steiner et al. (20) appear to contrast the findings of a more recent study in fibroblasts (37). It was reported that Rat1 cells infected with a p27 retrovirus had inactive cyclin E/cdk2 complexes and arrested in G_1. Co-expression of Myc with p27 promoted cdk2 activation and released the cells from the G_1 arrest without altering the p27 protein levels. The authors proposed that Myc indirectly promoted the sequestration and inactivation of p27.

Rb and the G_1 cyclin dependent kinases

In its hypophosphorylated state, the retinoblastoma protein prevents cells from exiting the G_1 phase of the cell cycle (reviewed in 38). Normally, as cells progress through G_1, Rb becomes increasingly phosphorylated, allowing the cells to proceed into S phase to complete the rest of the cycle.

The high levels of phosphorylated Rb in A1N4-myc cells was most likely due to the elevated cdk2 activity, which was significant even in arrested cells. *In vitro*, several cyclin/cdk complexes can phosphorylate Rb, but *in vivo*, the mechanism of Rb phosphorylation is not fully understood. Both cyclin D- and cyclin E- associated kinases have been implicated in Rb phosphorylation (39-42), but the timing of Rb hyperphosphorylation in normal cells most closely corresponds with the activation of cyclin E/cdk2 (43-44). A recent study in Rat1a cells also points to an important role for cdk2 activity in Myc-driven cell cycle progression (45). The authors found that cyclin A expression following Myc induction could be blocked by microinjected expression plasmids encoding cdk inhibitors or kinase negative cdk2, or by treatment with a cdk2-specific chemical inhibitor.

Alternatively, the constitutively hyperphosphorylated state of Rb could be due to an inability of the A1N4-myc cells to dephosphorylate Rb. Normally, Rb is dephophorylated by protein phosphatase types 1 and 2 during mitosis (46, 47). It is difficult to distinguish between the two scenarios in our system, since c-*myc* expression is also constitutive. An inducible c-*myc* expression construct may provide a better system for distinguishing between the two possibilities.

It is also interesting to note that fibroblasts prepared from Rb knockout mouse embryos also exhibit a shortened G_1 phase compared to wild type fibroblasts, and like the A1N4-myc cells, the Rb deficient cells are still dependent on an external growth signal and can be arrested in G_1 by serum withdrawal (48). Furthermore, the Rb negative cells display premature and elevated expression of cyclin E, but comparatively insignificant changes (either quantitative or temporal) in the expression of several other cell cycle regulated genes, including cyclin D1. Those results

reiterate the likely connection between Rb function and cyclin E expression (and thus cdk2 activity).

Although cdk4 levels appeared to be elevated in A1N4-myc cells compared to parental cells, there was little difference in cyclin D1 induction following EGF stimulation, implying that altered cyclin D1-associated activity did not contribute significantly to the change in growth rate. Cyclin D1 expression was very low or undetectable in arrested cells and was rapidly induced by EGF stimulation in both cell lines. Those findings are in accord with the hypothesis that Myc and cyclin D1 function in complimentary, rather than linear pathways (49). In the Rat1a fibroblast system, it was also determined that c-*myc* overexpression did not eliminate the requirement of cyclin D1 induction by serum for cell cycle progression (20). Furthermore, since Myc appeared to induce phosphorylation of Rb prior to induction of either cyclin D1 expression or cdk4 activity in both parental and Myc-transfected Rat1a cells, those results suggest that cyclin D1-associated activity is necessary for some other aspect of G_1 progression. Recently, a novel target of cdk4 and cdk6 was identified in a human breast cancer cell line (50), and certainly there could be other, as yet undefined, targets of cyclin D1-associated kinases. Nonetheless, cyclin D1 is not required for cell cycle progression in some cells that are functionally deficient for Rb due to mutation or viral oncoprotein expression, (51), and cyclin D1 mRNA and protein expression is often low in breast cancer cell lines which lack Rb function (52). Perhaps the effect of constitutive phosphorylation of Rb is different from that of functional Rb inactivation by mutation, deletion, or association with viral oncoproteins, with regard to the cyclin D1 requirement in the cell cycle. It should also be noted that cyclin D1 binds to Rb through a domain similar to those found in viral oncoproteins which interact with and inactivate the tumor suppressor (53), suggesting that a physical interaction between the two proteins may lead to further inactivation of one or the other.

A role for Cdc25A?

The Cdc25 family of phosphatases have also been implicated in the regulation of cdk activity, since they remove inhibitory phosphate groups at serine 14 and tyrosine 15 on cdks (21). Although UV irradiation stimulates tyrosine phosphorylation of cdk4 with subsequent G_1 arrest

(54), a clear function for such a cdk4 species in normal cell cycle progression has not been demonstrated. Thus Cdc25 expression may not be as important for cyclin D1/cdk4 activity as it is for cdk2 activation.

The A and B forms of Cdc25 can function as transforming oncogenes in cooperation with activated Ha-ras or loss of Rb (55). The synergism between Ras and Cdc25 may be explained by the observation that Raf1, a component of the Ras pathway, can phosphorylate and activate Cdc25 proteins (56). That assertion could also explain the results of Steiner et al., (20). They found that full induction of cdk2 kinase activity by Myc also required Cdc25A activity, which could apparently be stimulated by serum growth factors, perhaps through the Ras-Raf pathway. A more recent study indicates that cdc25A expression can be directly induced by Myc in fibrobasts (22). However, another study using a similar rat fibroblast cell line showed no increase in Cdc25A steady state levels when Myc was overexpressed (37). In our MEC system, cdc25A RNA levels were elevated by Myc overexpression, but the timing of cdc25A expression induction following exposure to EGF was quite similar in the two MEC lines. The RNA was first detected 12 hours after EGF addition, suggesting that other factors in addition to Myc are required for cdc25A expression.

Distinguishing the roles of Myc in cell cycle, apoptosis, and malignant progression

In contrast to fibroblast models in which c-*myc* expression was sufficient to force quiescent cells to re-enter the cell cycle (12, 13), c-*myc* overexpression was not sufficient to drive the mammary epithelial cells through the cell cycle in the absence of a growth stimulus (EGF). That difference may simply be due to cell type specificity or experimental conditions, but it should also be pointed out that although the fibroblasts re-entered the cell cycle, they were executing an apoptotic pathway rather than an actual proliferative response. The A1N4-myc cells, like the parental A1N4 cell line, reversibly arrest in G_1 in the absence of EGF, rather than undergoing apoptosis. Deregulated c-*myc* expression can induce apoptosis in primary mouse tumor MECs in the absence of growth/survival factors (28, 29). Thus, the results suggest that A1N4 cells, presumably during the process of immortalization, have undergone some change which makes

them incapable of executing the apoptotic pathway in response to Myc. Although mutation of p53 is an attractive postulation to account for the difference, no mutations were found in the highly conserved exons 4-9 in the immortalized cell line (57). However, it was noted in that study that p53 proteins levels were unusually high, suggesting that the protein may be posttranscriptionally modified. Whatever the cause, the end result is that these cell lines provide an excellent model for studying alterations in cell cycle control due to c-*myc* overexpression in the absence of the confounding effects of apoptosis induction. That is an important distinction to make since a recent study found that the effects of Myc on cell cycle progression and apoptosis are indeed distinct (45).

In summary, our results may provide at least a partial explanation as to why Myc and EGF can cooperate to transform MECs and similarly, why there is such a strong synergism between Myc and TGFα in mammary tumorigenesis, as demonstrated by transgenic mouse models. Myc overexpression, in conjunction with EGF receptor stimulation, forces the cells through G_1 at a faster rate, resulting in accelerated growth. If this phenotype also allows epithelial cells within the mammary gland to proliferate under some conditions which would normally induce a G_1 arrest and block DNA replication, increased genetic instability may also be a logical endpoint, analogous to the phenomenon which was demonstrated for p53 mutations (58, 59). Indeed, it has already been demonstrated that prolonged Myc overexpression in Rat1a cells can promote a variety of genetic aberrations, including numerical changes, chromosome breakage, and the formation of circular chromosomal structures, chromosome fusions, and extrachromosomal elements (60). In further support of that hypothesis, a recent study demonstrated that ectopic expression of p27 (which was down-regulated in the A1N4-myc cells) suppressed tumor growth and the accumulation of aneuploid cells in a brain tumor model (61).

Materials and Methods

Cell lines

A pair of human mammary epithelial cell lines (184A1N4, 184A1N4-myc) were used to study the effects of c-*myc* overexpression on cell cycle regulation. The parental cell line, A1N4, was derived from normal mammary tissue obtained by reduction mammoplasty and was immortalized with benzo(a)pyrene (62). The A1N4-myc line (27) was established *via* retroviral infection of A1N4 cells with a construct containing mouse c-*myc* under the control of the Moloney mouse leukemia virus long terminal repeat (MMLV LTR). Both cells lines were maintained in IMEM (Gibco-BRL, Gaithersburg, MD) containing 0.5% FCS, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin (Biofluids, Rockville, MD), and 10 ng/ml EGF (Upstate Biotechnology Incorporated [UBI], Lake Placid, NY). The cells arrest in G_1 in the absence of EGF (63).

A pair of mouse mammary cell lines (HC14 and HC14-myc) was also used in preliminary experiments. The HC14 line was established from a mid-pregnant mammary gland and was transfected with a c-*myc* expression construct driven by the MMLV LTR (64). Both cell lines were routinely grown in IMEM with 10% FCS.

Growth assays

Cells were plated in 96-well plates (Costar, Cambridge, MA) at a density of 1000-2000 cells/well. At various time points, plates were stained with crystal violet (Sigma, 0.5% in 30% MeOH), rinsed with water and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc.). Doubling times were calculated from the slope of the line generated by plotting log(absorbance) vs time.

FACS Analysis

Cells were plated ($5x10^5$ cells/plate) in 10 cm dishes (Falcon 3003, VWR Scientific, Philadelphia, PA) in normal growth media containing EGF. The next day the cells were changed to EGF-free media to arrest them in G_1. After 48 hours, the cells were restimulated with EGF (10

ng/ml) and cells were harvested at 3 h intervals. Nuclei were isolated and stained with propidium iodide for cell cycle analysis according to the method of Vindelov et al. (65).

Western Analysis

Cells were plated, arrested, and restimulated with EGF as described for FACS analysis. At 1.5 or 3 hour intervals following EGF stimulation, total cell lysates were prepared. Cells were washed with cold PBS and then scraped into cold lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton x-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3O_4V, 100 mM NaF, 10 mM pyrophosphate, 10 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). After a 10 minute incubation on ice, lysates were spun for 10 minutes in a cold micocentrifuge to remove cellular debris and were frozen at -70 °C. Twenty µg of protein from each sample were separated by SDS-PAGE and transferred to either nitrocellulose or PVDF membranes. Acrylamide concentrations varied depending on the target protein as follows; Rb, 6%; cyclin D1, cyclin E, cdk2, and cdk4, 10%; p21 and p27, 14%. Blots were blocked in 4% milk, 1% BSA in Tris buffered saline with Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween-20) for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (1 µg/ml): Rb (Pharmingen, San Diego, CA), cyclin D1 and E (UBI), cdk2 and cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or p27 (Santa Cruz). Proteins were visualized with an HRP-linked second antibody (1/2000 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce, Rockford, IL). Amido black or India ink staining of the membranes demonstrated equal loading and transfer of the samples. Since appropriate antibody for Cdc25A was not commercially available, we chose to examine its expression at the RNA level only (see below).

Kinase assays

Cell lysates (100 µg) were incubated with 1 µg anti-cdk2 antibody for 2 h (4°C) prior to precipitation with Agarose A beads (Santa Cruz). Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 1 mM DTT, 0.3 mM ß-glycerophosphate, 1 mM Na_3O_4V, 10 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). The beads were then resuspended in 30 µl kinase buffer and the

reaction was started by adding ATP (200 μM), γ^{32}P-ATP (5 μCi) and histone H1 (1 μg). Samples were incubated at 30 °C for 15 min before stopping the reaction with 2x loading buffer (62.5 mM Tris [pH 6.8], 10% sucrose, 2% SDS, 5% ß-mercaptoethanol, 1% bromphenol blue). Labeled proteins were run on a 10% polyacrylamide gel which was dried prior to visualization with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale CA).

RNA Isolation

A1N4 and A1N4-myc cells were plated sparsely (1.5 x10^6 cells) in culture flasks (225 cm^2; Costar) and growth arrested as described above. Following re-stimulation with EGF (10 ng/ml), total RNA was harvested at three hour intervals by the guanidine thiocyanate-acid phenol method (66).

Northern Analysis

Total RNA (12 μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were hybridized overnight with a 700bp ^{32}P-labeled, random-primed human probe for cdc25A (nt 936-1637). Bands were detected with a PhosphorImager 445 SI.

RNase Protection Assay

Changes in cyclin RNA expression were examined using a nonradioactive RNase protection assay, as previously decribed (31). A pGEM-4z vector containing a 400 bp fragment of the human cyclin A cDNA was linearized with EcoRI prior to synthesis of a 440 bp riboprobe. A 1.3 kb NotI fragment of human cyclin D1 in a Bluescript KS- plasmid was linearized with EcoNI to synthesize a 360 bp probe.

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Footnote

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Cell Line	Doubling Time	Difference
A1N4	27.4 +/-0.6 h	5.9 h
A1N4-myc	21.5 +/-0.3 h	
HC14	25.1 +/- 0.5 h	6.3 h
HC14-myc	18.8 +/-0.3 h	
MMEC[1]	24.0 h	5.8 h
MMEC-myc[1]	18.2 h	

Table 1: Doubling times for 1 human pair and 2 mouse pairs of cell lines, +/- S.E. (pair=c-*myc*-overexpressing line and its parental line). The last column indicates the decrease in doubling time of the Myc line compared to its parental line.

[1]Reference 26.

Figure Legends

Figure 1: Growth of A1N4 and A1N4-myc cells in response to EGF is concentration dependent. Cells were plated in 96 well plates with increasing concentrations of EGF and incubated for three days before being stained with crystal violet. Note that the two curves are parallel. n=8, +/- S.E.

Figure 2: A1N4 and A1N4-myc cells arrest in G_1 in the absence of EGF.
A: Growth of both A1N4 and A1N4-myc cells is dependent on EGF. Cells were plated in 96 well plates (10^3 cells/well) in the presence or absence of EGF and were stained with crystal violet at the indicated times. Relative cell number was then measured as absorbance at 540 nm. n=8. **B:** Cell cycle histograms for unsynchronized cells grown in normal media with EGF and arrested A1N4 and A1N4-myc cells which had been deprived of EGF for 48 h.

Figure 3: Cell cycle analysis of A1N4 and A1N4-myc cells re-stimulated with EGF following growth arrest for 48 hours. Arrested cells were treated with 10 ng/ml EGF and harvested at 3 hour intervals. Propidium iodide staining and FACS analysis was performed with isolated nuclei.

Figure 4: Expression and phosphorylation of Rb in synchronized cells (A1N4 and A1N4-myc). Arrested cells were re-stimulated with EGF and whole cell lysates were prepared at the times indicated. 20 μg of protein were separated on a 6% gel before transfer to nitrocellulose for Western analysis. The faster moving band is due to hypophosphorylated (inhibitory) Rb and the upper band contains hyperphosphoylated Rb. A0=A1N4 at time 0. M0=A1N4-myc at time 0, +=unsynchronized cells.

Figure 5: Expression of the G_1 cyclins D1 and E, their associated kinases ckd4 and cdk2, and the cdk inhibitor p27 in synchronized A1N4 and A1N4-myc cells. Lysates were harvested as in Figure 4 and were separated on 10% acrylamide gels prior to transfer to nitrocellulose for western analysis. In the case of cdk2, phosphorylation by CAK leads to a downward shift in mobility, producing the observed doublet. +, unsynchronized cells.

Figure 6: Kinase activity of cdk2 in synchronized A1N4 and A1N4-myc cells. Cdk2 was immunoprecipitated from whole cell lysates at the indicated times following EGF re-stimulation. The precipitates were then incubated for 15 min at 37 °C in the presence of histone H1 and γATP. Labeled substrate was detected by phosphorimager analysis following fractionation on a 10% PAGE gel.

Figure 7: Northern analysis of cdc25A RNA in A1N4 and A1N4-myc cells. **A**: Expression in unsynchronized cells. Cells were grown in the presence of EGF and were harvested at approximately 75% confluence. **B**: Cell cycle dependent expression. Cells were arrested and re-stimulated by addition of EGF as in Figure 3. At the times indicated, total RNA was harvested.

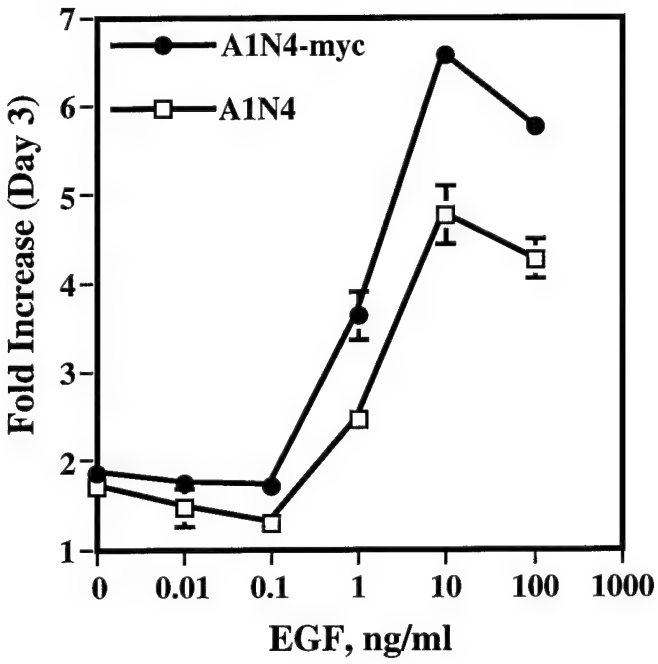

A1N4-myc
A1N4
Fold Increase (Day 3)
7
6
5
4
3
2
1
0
0.01
0.1
1
10
100
1000
EGF, ng/ml

Figure 1

Figure 2

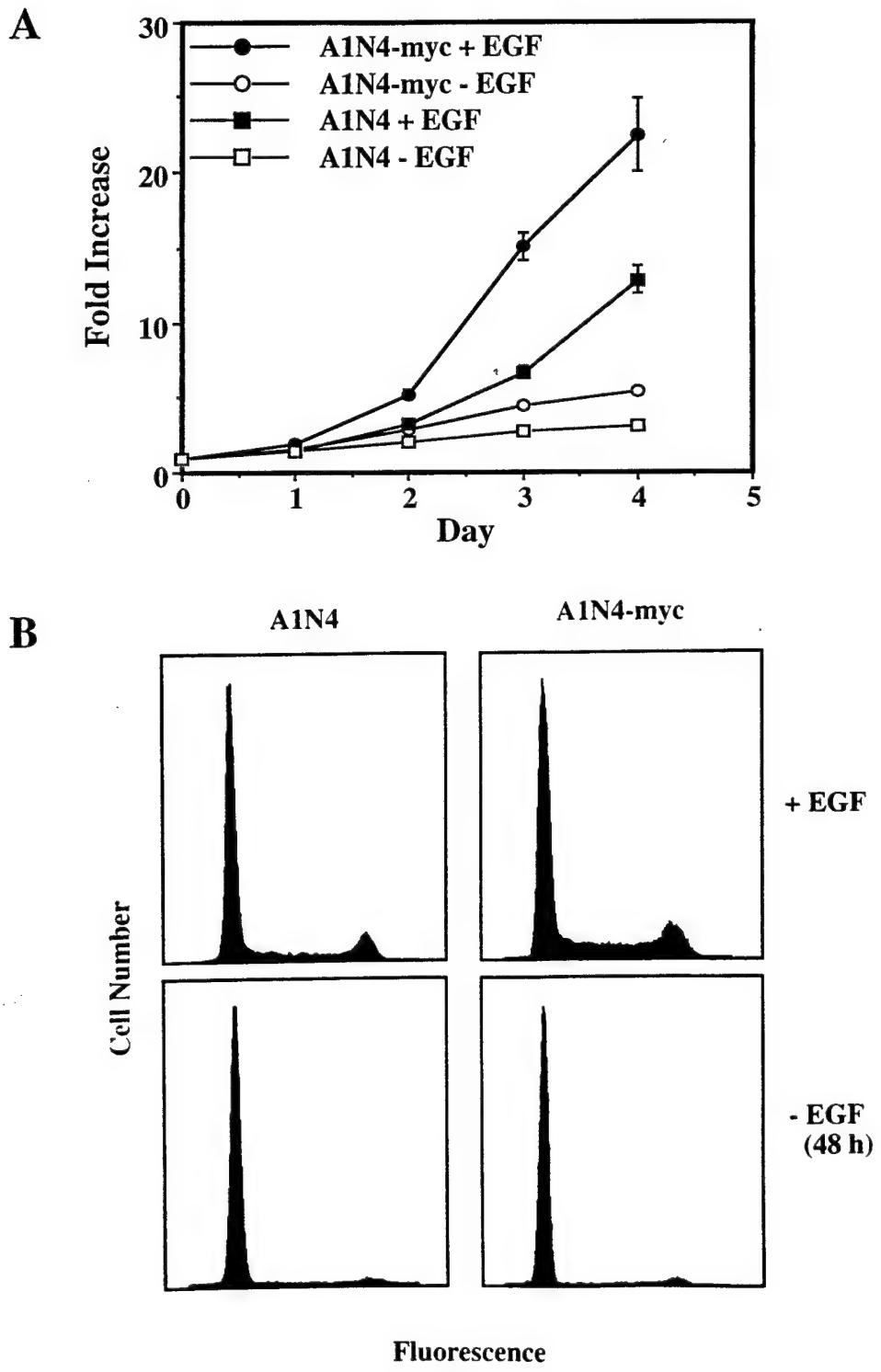

A
30
20
10
0
Fold Increase
A1N4-myc + EGF
A1N4-myc - EGF
A1N4 + EGF
A1N4 - EGF
0
1
2
3
4
5
Day
B
A1N4
A1N4-myc
+ EGF
- EGF
(48 h)
Cell Number
Fluorescence

Figure 3

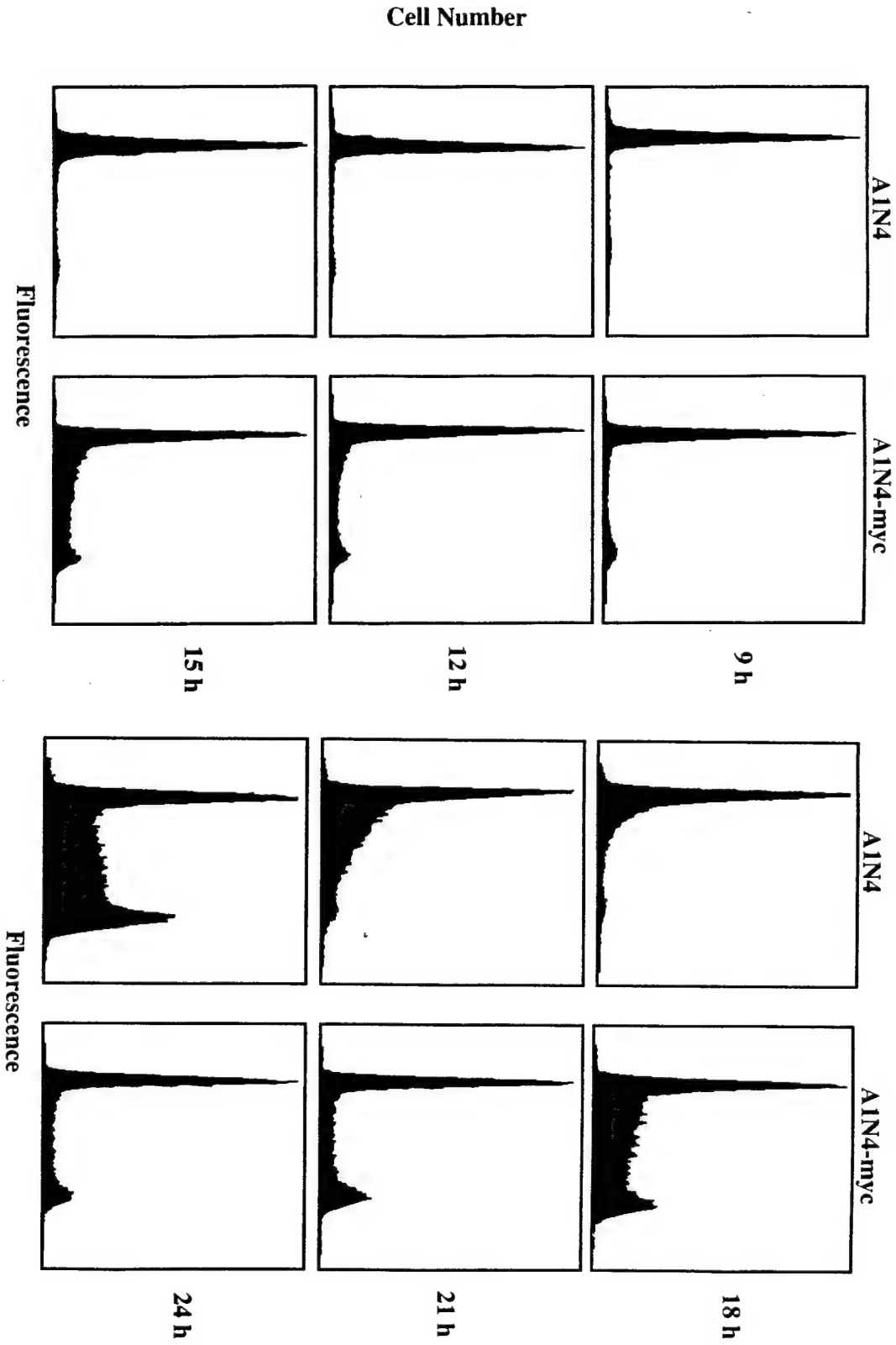

Cell Number
A1N4
A1N4-myc
9 h
12 h
15 h
18 h
21 h
24 h
Fluorescence
Fluorescence

Figure 4

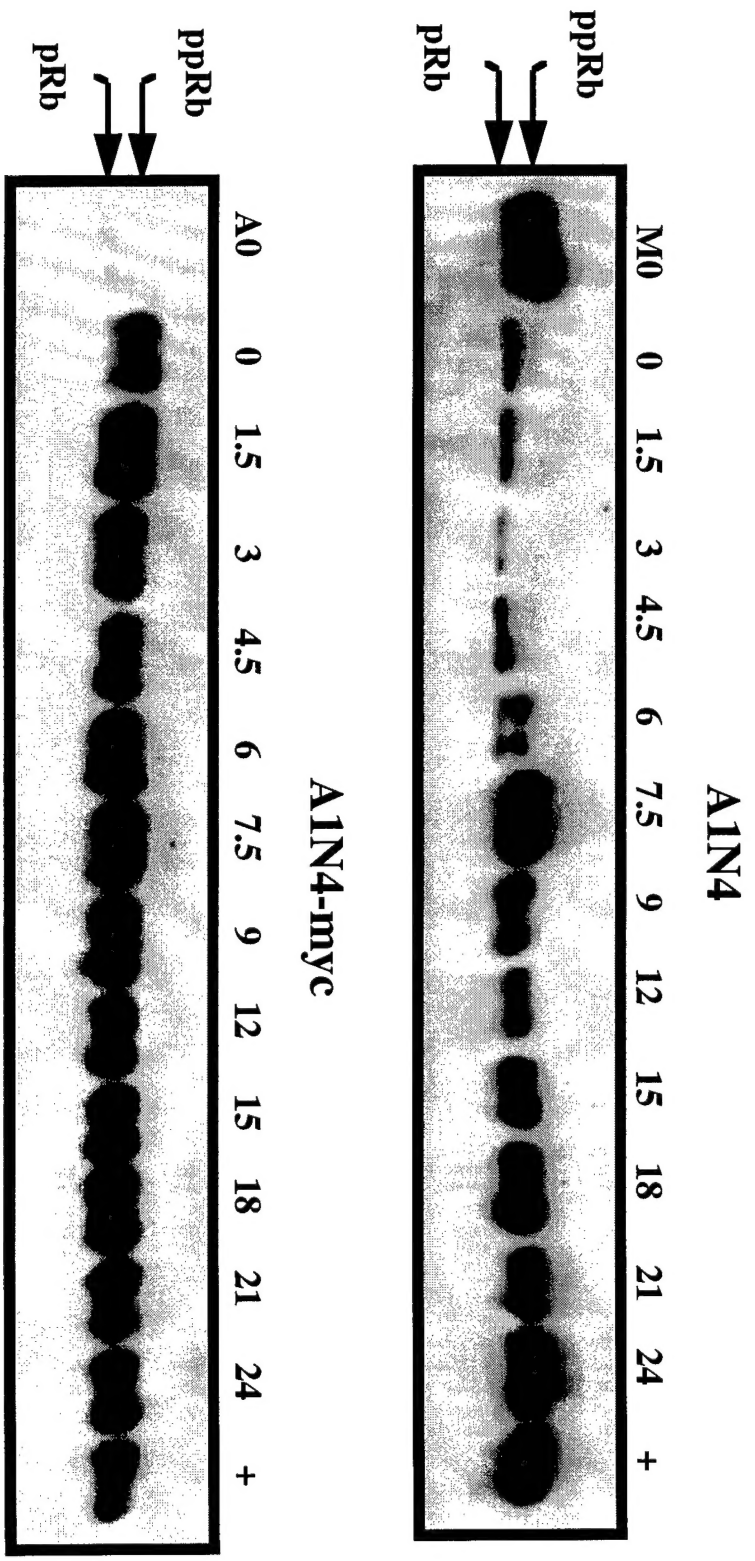
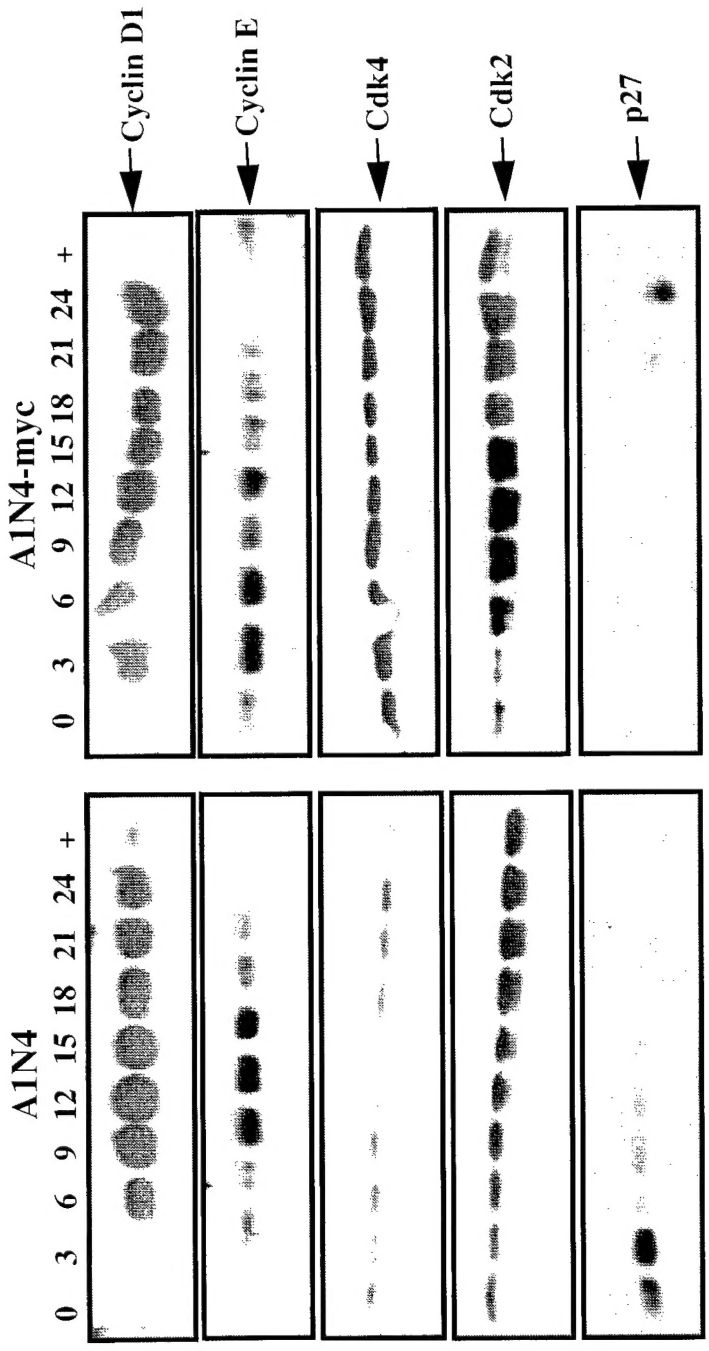

A1N4
ppRb
pRb
M0 0 1.5 3 4.5 6 7.5 9 12 15 18 21 24 +
A1N4-myc
ppRb
pRb
A0 0 1.5 3 4.5 6 7.5 9 12 15 18 21 24 +

Figure 5


Cyclin D1
Cyclin E
Cdk4
Cdk2
p27
A1N4-myc
0 3 6 9 12 15 18 21 24 +
A1N4
0 3 6 9 12 15 18 21 24 +

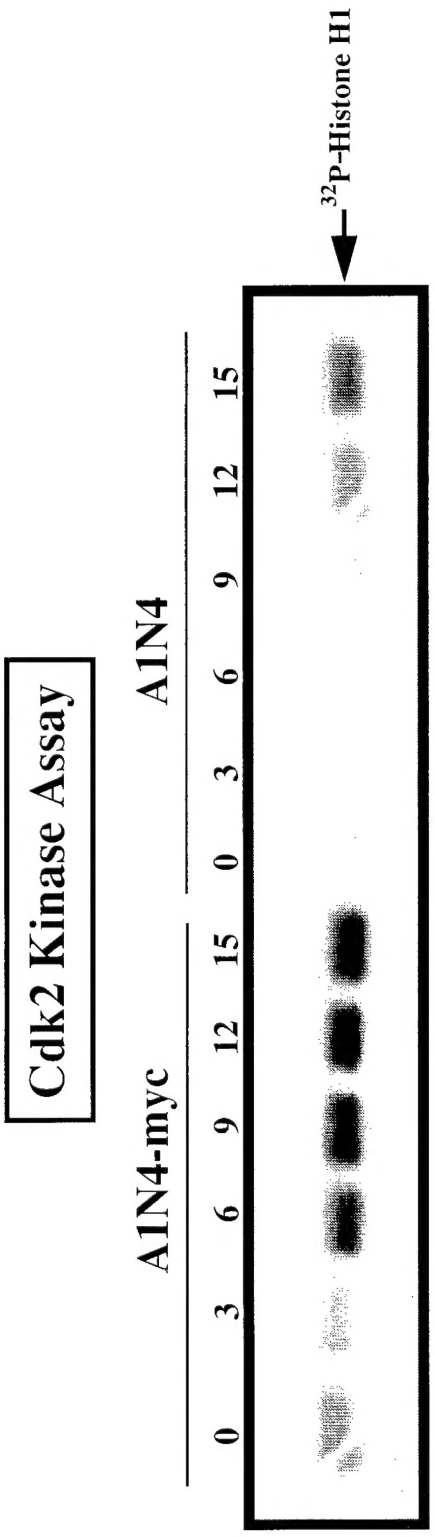
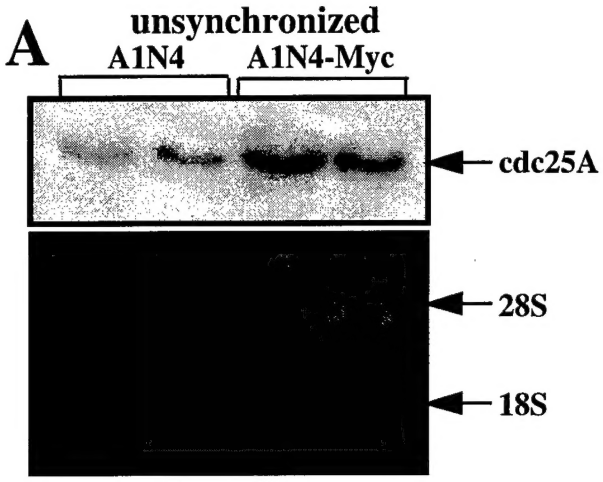
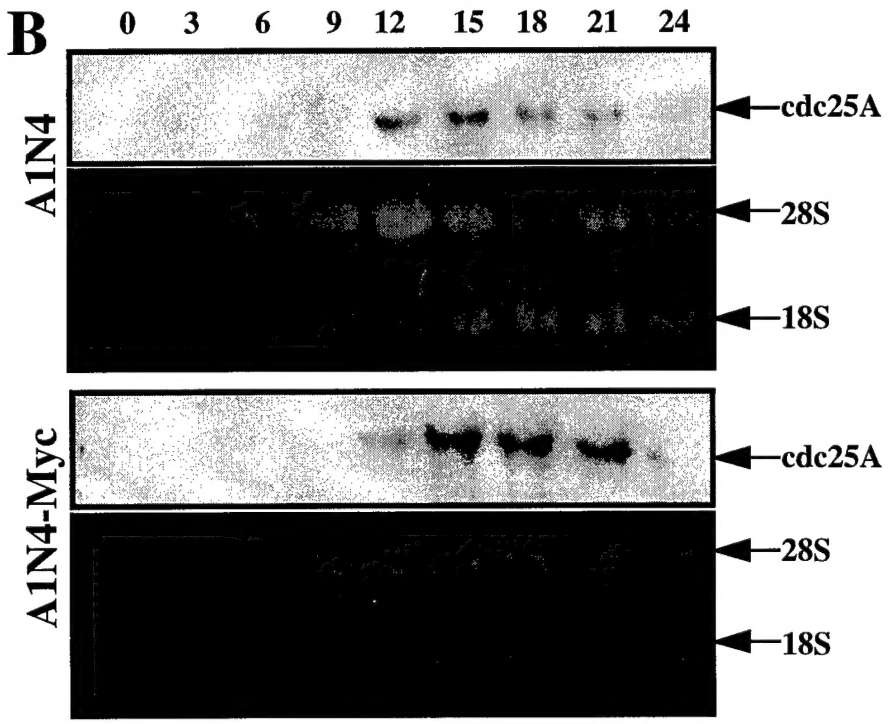

Cdk2 Kinase Assay
A1N4-myc
0 3 6 9 12 15
A1N4
0 3 6 9 12 15
32P-Histone H1

Figure 6

Figure 7


A
unsynchronized
A1N4
A1N4-Myc
cdc25A
28S
18S


B
0 3 6 9 12 15 18 21 24
A1N4
cdc25A
28S
18S
A1N4-Myc
cdc25A
28S
18S